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Sensitive Immunoassays for the Measurement of Human Lysozyme

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Three immunological methods (radioimmunoassays and enzymoimmunoassay) were established for the sensitive and specific measurement of human lysozyme in biological fluids. Using these methods, human lysozyme was determined in the concentration range of 5 to 250 ng/ml. Although radioimmunoassay with dextran-coated charcoal offered the best precision, enzymoimmunoassay by the sandwich technique using microtiter plates was found to be the most convenient method, giving satisfactory precision and reproducibility.

Keywords——human lysozyme; radioimmunoassay; enzymoimmunoassay; sandwich technique; lysozyme level of normal human serum

Lysozyme activity in various tissues and fluids is known to vary with physiological or pathological conditions. The activity, therefore, has been used by many investigators as an index of disease states in the fields of hematology,[2] oncology,[3] nephrology[4] and gastro enterology.[5] In these studies, assay was based on the enzymatic activity of lysozyme with *M. lysodeikticus* as a substrate. Since lysozyme activity is affected by many factors,[6] the values of activity obtained by different investigators have not coincided, and their diagnostic interpretations have differed. In order to assess the diagnostic usefulness of lysozyme, it is necessary to develop a specific and sensitive method for the determination of lysozyme.

This report deals with an investigation of three immunological assay methods for human milk lysozyme; radioimmunoassay by the competitive and sandwich techniques, and enzymoimmunoassay by the sandwich technique. The reactivities of three lysozymes originating from leucocytes, placenta and leukemia urine with the antibody against milk lysozyme were investigated.

Materials and Methods

Human Lysozymes——Human milk lysozyme (HML), human leucocytes lysozyme (HLL) and human placenta lysozyme (HPL) were prepared by the methods described previously,[7] and human leukemia urine

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lysozyme (HUL) was kindly provided by Dr. E.F. Osserman, Columbia University, New York. All four lysozymes were homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A stock solution of HML was prepared with 0.05 M Tris/HCl buffer (pH 7.4) and the concentration was calculated based on the absorbance at 280 nm (2.55 at 1 mg/ml).[8] The stock solution was diluted with the buffer containing 0.2% bovine serum albumin (BSA) and 0.15 M NaCl.

Antiserum and Antibody——Two to 3 mg of HML in 1 ml of 0.15 M NaCl was mixed with 1 ml of Freund's complete adjuvant (Difco Lab.) to form a water-in-oil emulsion. Two ml of the emulsion was injected into the hind foot pads and femoral hypodermis of a rabbit weighing about 2 kg. The injection of the emulsion was repeated 3, 5 and 7 weeks after the first immunization. One week after the final immunization, 2 to 3 mg of HML in 1 ml of 0.15 M NaCl was injected into the ear vein. The antiserum against HML was obtained from the vein one week later.

The antibody against HML was purified to the IgG fraction by an immunoadsorbent technique. Thirty ml of the antiserum was applied to a column (1×5 cm) of HML-coupled Sepharose 4B, and the adsorbed fraction was eluted with 0.17 M glycine/HCl buffer, pH 2.3. The fraction was dialyzed against distilled water and lyophilized (49.5 mg); this was used as the specific antibody.

Preparation of Radioactive Human Milk Lysozyme——^{125}I-Labeled HML was prepared by the chloramine-T method[9] as described previously.[10] One to 2 mCi of Na^{125}I (5 mCi/50 μl; The Radiochemical Centre, England) was reacted with 6 to 10 μg of HML. The radiochemical yield of ^{125}I-labeled HML was 51.3% on paper chromatography with 95% ethanol-2 M ammonia (9: 1, v/v) as a solvent. ^{125}I-Labeled HML was purified on a column (1.5×30 cm) of Sephadex G-25 in 0.05 M sodium phosphate buffer (pH 7.4) containing 0.2% BSA and 0.15 M NaCl; its specific radioactivity was 73—122 mCi/mg. The fraction of ^{125}I-labeled HML was diluted to 1×10^5 cpm/ml with the buffer described above.

Preparation of Radioactive Antibody——Two mg of the antibody was reacted with 0.5 mCi of Na ^{125}I in 0.4 ml of 0.5 M sodium phosphate buffer (pH 7.4) by the chloramine-T method.[9] The reaction mixture was fractionated with a column (1.5×30 cm) of Sephadex G-25. The specific radioactivity of ^{125}I-labeled antibody was 125 μCi/mg. The fraction containing radioactive antibody was diluted at 1: 101 with 0.05 M sodium phosphate buffer (pH 7.4) containing 0.2% BSA and 0.15 M NaCl.

Conjugation of Alkaline Phosphatase with the Antibody——One mg of alkaline phosphatase (1025 unit/mg, from calf intestine; type VII, Sigma) was mixed with 1.6 mg of the antibody in 0.4 ml of 0.05 M Tris/HCl buffer (pH 8.6), then 20 μl of 2.5% (v/v) glutardialdehyde was added to the mixture. After 5 min at room temperature, the antibody conjugated to alkaline phosphatase (AP-antibody conjugates) was dialyzed against 0.05 M Tris/HCl buffer (pH 8.6) and diluted at 1: 101 with 0.05 M Tris/HCl buffer (pH 7.4) containing 0.2% BSA and 0.15 M NaCl.

Dextran-coated Charcoal and Microtiter Plates——Dextran-coated charcoal was prepared according to the method of Herbert *et al.*[11] A 10% charcoal ("Norit A," American Norit Co.) suspension was mixed with an equal volume of 1% dextran T-70 (Pharmacia) solution in 0.05 M sodium phosphate buffer (pH 7.4) containing 0.2% BSA and 0.15 M NaCl.

Disposable microtitration plates (microtiter plates) made of flexible polyvinyl chloride were obtained from Cooke Lab. The plate had 96 wells and the capacity of each well was 0.27 ml.

Determination of Radioactivity and Alkaline Phosphatase Activity——The radioactivity was measured in a well-type scintillation counter (Aloka JDC-207). The radioactivity counting efficiency was 70%. Alkaline phosphatase activity was measured by the reported method.[12] The substrate solution was 0.5% (w/v) Na *p*-nitrophenylphosphate (Sigma 104®) in 0.05 M glycine/NaOH buffer, pH 10.5. The amount of AP-antibody conjugates bound to HML in microtiter plate was determined after adding 0.2 ml of the substrate solution. After 30 min at 37°, the reaction was stopped by adding 0.1 ml of 0.1 N NaOH and the mixture was diluted to 1.8 ml with 0.02 N NaOH. The absorbance at 405 nm was measured in 1 cm microcuvette using a Hitachi 156 spectrophotometer.

Results

Radioimmunoassay of Human Lysozyme by the Competitive Technique

Radioimmunoassay of human lysozyme by the competitive technique was performed according to the procedure for radioimmunoassay of hen egg-white lysozyme as described previously.[10] In a 10 ml test tube, 0.1 ml of ^{125}I-labeled HML (10000 cpm) and 0.1 ml of

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unlabeled HML were mixed with 0.1 ml of antiserum diluted at 1: 20000. The mixture was incubated at 37° for 2 hr and then 0.5 ml of dextran-coated charcoal was added. After centrifugation at 3000 rpm for 15 min, the supernatant was decanted into a counting tube and the radioactivity was assayed.

As shown in Fig. 1, the recovery of labeled HML in the supernatant decreased with increasing concentration of unlabeled HML. In this procedure, 2.5 to 100 ng/ml of HML was determinable.

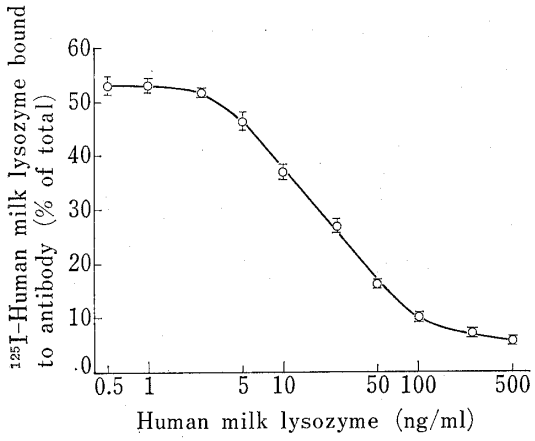

Fig. 1. Standard Curve for the Measurement of Human Milk Lysozyme by the Competitive Technique (Dextran-coated Charcoal) with ^{125}I-Labeled Human Milk Lysozyme

Each point represents the mean ± S.D. of five experiments.

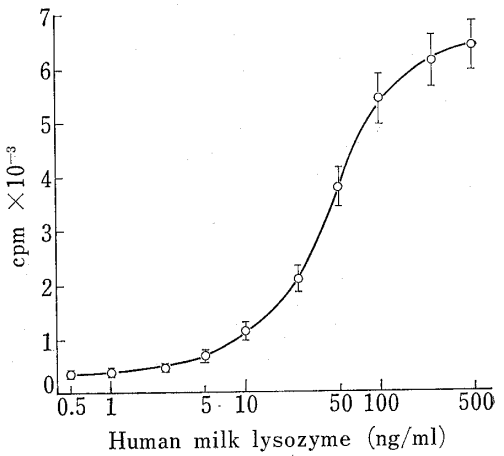

Fig. 2. Standard Curve for the Measurement of Human Milk Lysozyme by the Sandwich Technique (Microtiter Plates) with ^{125}I-Labeled Antibody

Each point represents the mean ± S.D. of five experiments.

Radioimmunoassay of Human Lysozyme by the Sandwich Technique

A more convenient method than radioimmunoassay with ^{125}I-labeled antigen was investigated. Instead of labeling the antigen, the antibody against HML was labeled with ^{125}I. One-tenth ml of HML standard was poured into the wells of a microtiter plate which had first been coated with 10 μg/ml of the antibody, and the plate was incubated at 37° for 1 hr. After washing the wells, 0.1 ml of ^{125}I-labeled antibody in 1: 101 dilution was poured into the wells and incubated at 37° for 1 hr. After further washing, 0.2 ml of 0.1 N NaOH was poured into the wells, then the radioactivity of the NaOH solution was measured.

As shown in Fig. 2, the radioactivity of antibody bound to antigen increased with increasing concentration of HML. In this procedure, 5 to 250 ng/ml of HML was determinable. This method was found to be more convenient than the method with ^{125}I-labeled antigen.

Enzymoimmunoassay of Human Lysozyme by the Sandwich Technique

In order to avoid the use of radioisotopes, an enzymoimmunoassay by the sandwich technique was investigated.

As shown in Table I, HML was first reacted with antibody which had been adsorbed on the bottom of the wells (step 2 in Table I), then the HML-antibody complex was reacted with AP-antibody conjugates (step 3) to form a sandwich of HML between the antibody and AP-antibody conjugates. After washing out free AP-antibody conjugates, alkaline phosphatase activity remaining in the wells was measured (step 4). The relation between the alkaline phosphatase activity and the concentration of HML is shown in Fig. 3A.

The phosphatase activity (A_{405} nm) remaining in the wells increased with increasing concentration of HML. In this procedure, 5 to 250 ng/ml of HML was determinable. The reactivity of HLL, HPL or HUL with the antibody against HML was estimated by enzymo-

Table I. Standard Procedure for the Enzymoimmunoassay of Human Lysozyme by the Sandwich Technique

1.	Antibody against HML (10 μg/ml) incubate overnight at 4° in the wells of a microtiter plate and wash the wells four times with 0.2 ml of H_2O.	0.15 ml
2.	Human lysozyme standard or samples incubate at 37° for 1 hr and wash the wells four times with 0.2 ml of buffer[a]	0.1 ml
3.	Alkaline phosphatase-antibody conjugates of 1: 101 dilution incubate at 37° for 1 hr and wash the wells four times with 0.2 ml of buffer.[a]	0.1 ml
4.	Na *p*-nitrophenylphosphate (5 mg/ml) incubate at 37° for 30 min	0.2 ml
5.	0.1 N NaOH	0.1 ml
6.	Measure A_{405} nm in 1.8 ml diluted with 1.5 ml of 0.02 N NaOH	

a) Buffer; 0.05 M Tris/HCl buffer (pH 7.4) containing 0.2% bovine serum albumin and 0.15 M NaCl.

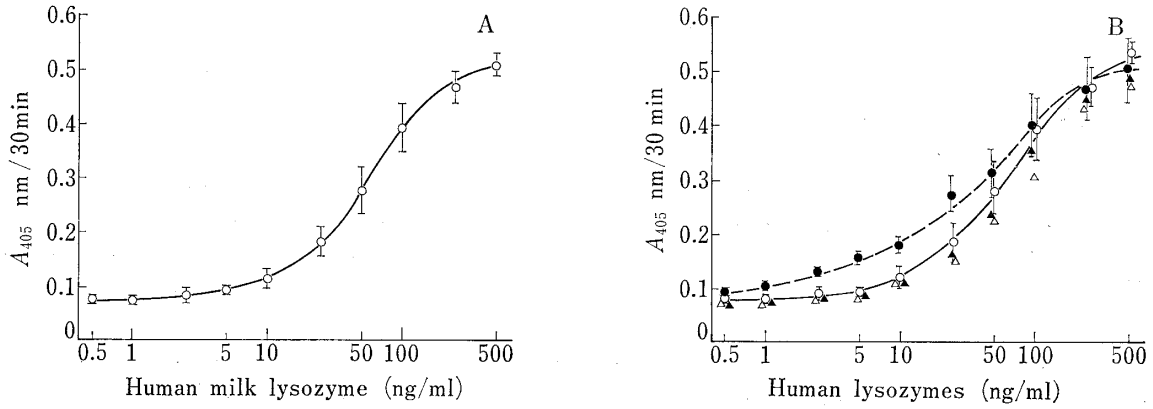

Fig. 3. Standard Curve for the Measurement of Human Hilk Lysozyme by the Sandwich Technique (Microtiter Plates) with Alkaline Phosphatase-Antibody Conjugates (Fig. 3A), and Comparison of the Reactivities of Four Human Lysozymes, HML (○), HUL (●), HPL (△) and HLL (▲), with the Antibody against Human Milk Lysozyme (Fig. 3B)

Each point in Fig. 3A represents the mean ± S.D. of five experiments and each point in Fig. 3B represents the mean ± S.D. of three experiments.

immunoassay, as shown in Fig. 3B. The curves of HLL and HPL were similar to that of HML, but the curve of HUL was slightly different.

Precision of Assay by the Three Methods

The precision in the measurement of human lysozyme by the three methods was estimated from the results of five successive analyses of various concentrations of HML. The mean standard deviations corresponded to ±5.4% in radioimmunoassay with dextran-coated charcoal, ±13.0% in radioimmunoassay with microtiter plates and ±12.4% in enzymoimmunoassay with the plates. The assay with dextran-coated charcoal was thus the most precise.

Determination of Lysozyme Level in Human Serum

The lysozyme level of normal human serum was determined by enzymoimmunoassay. A sample of 20 μl of serum was diluted at 1: 32 with 0.05 M Tris/HCl buffer (pH 7.4) containing 0.2% BSA and 0.15 M NaCl, and 0.1 ml of diluted serum was used. The mean level (HML eq. μg/ml) with the S.D. in 11 human sera was 0.88±0.16, which corresponded to 0.43±0.13 HUL eq. μg/ml.

Discussion

A radioimmunoassay method for human lysozyme by a competitive technique was established. In this method, treatment with dextran-coated charcoal was necessary for the separation of free HML from antibody bound HML. The radioimmunoassay with dextran-

coated charcoal was precise, but had the following drawbacks: 1) a large amount of Na ^{125}I, 1—2 mCi, was required to prepare ^{125}I-labeled antigen; 2) ^{125}I-labeled antigen had to be used for the radioimmunoassay within two weeks after labeling; if left longer, it had to be purified again before use.

In order to overcome these difficulties, a method for radioimmunoassay with ^{125}I-labeled antibody was established and evaluated. It was found to be better than the method with ^{125}I-labeled antigen in the following respects: 1) only 0.5 mCi of Na ^{125}I was required to prepare ^{125}I-labeled antibody; 2) ^{125}I-labeled antibody could be used for the radioimmunoassay for at least two months after labeling; 3) the radiochemical purity of ^{125}I-labeled antibody did not affect radioimmunoassay by the sandwich technique.

An enzymoimmunoassay with AP-antibody conjugates was also established. HML at a concentration of 5 to 250 ng/ml could be determined by this method with better precision than by the method using a polystyrene test tube coated with IgG.[13] The enzymoimmunoassay does not require any radioisotope, and the AP-antibody conjugates can be used for at least 6 months. Thus, the enzymoimmunoassay of human lysozyme by the sandwich technique using microtiter plates was found to offer satisfactory precision, reproducibility and simplicity. As shown in Fig. 3B, the curves of HML, HPL and HLL were identical with each other, but that of HUL was different. The difference in the curve for HUL was also observed by the competitive technique with ^{125}I-labeled HML and by the sandwich technique with ^{125}I-labeled antibody (data not shown). This result indicates that the immunoassay methods for HML can measure the levels of HML, HPL and HLL using a standard solution of HML, but cannot accurately measure the level of HUL without a standard solution of HUL.

The enzymoimmunoassay for HML gave a serum level of lysozyme in eleven human sera of 0.88±0.16 HML eq. μg/ml. The coefficient of correlation between the lysozyme level measured by enzymoimmunoassay and that measured by radioimmunoassay was 0.938 in the eleven human sera. The serum levels of lysozyme obtained by both methods were in good agreement, and were similar to those of Canfield *et al.*[8] who established a radioimmunoassay procedure for HUL by the competitive technique. It is important to identify the origin of human lysozyme used as a standard when measuring lysozyme levels in human materials by immunoassays, since the results reported here indicate that the origin of lysozyme affects the serum lysozyme level.

The lysozyme levels in various tissues and fluids have been determined as an index of disease states,[2—5] but the analytical method used in these studies was based on the bacteriolytic activity of lysozyme with *M. lysodeikticus* as a substrate. Therefore, further investigations are needed to compare the lysozyme levels obtained by the immunological methods and by the bacteriolytic method. Some preliminary results have been resported.[14]

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